NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

EVALUATION OF INNATE IMMUNE BIOMARKERS IN SALIVA FOR DIAGNOSTIC POTENTIAL OF BACTERIAL AND VIRAL RESPIRATORY INFECTIONS

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Declaration of Interest

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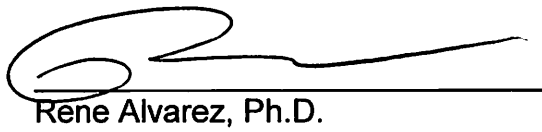

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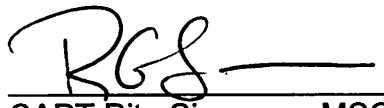

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Executive Summary

Background: Military group housing, training facilities, and operational theatres, combined with high stress, presents unique environments for dissemination and propagation of transmitting bacterial and viral infections. While often associated with mild illness, severe disease may occur with significant morbidity, leading to a detrimental impact on training schedules and operational readiness. Current diagnosis and monitoring of infections require invasive procedures by skilled technicians, including repeated blood draws, making it difficult for in-theatre care. Therefore, there remains a critical need for a rapid, sensitive assay for detection and diagnosis of microbial infections in our warfighters, both in garrison and in theatre.

Methods: In this study, we explored the presence of innate immune biomarkers in saliva associated with bacterial and viral respiratory infections, as compared to markers present in serum samples. A panel of 28 cytokines and chemokines in saliva and serum obtained from 38 healthy subjects and 19 bacterially infected or virally infected individuals were analyzed via bio-plex analysis.

Results: A unique set of innate immune biomarkers, including: IL-8, IL-9, IL-12, IL-13, eotaxin and IFNa2 were identified in saliva from infected patients allowing for differentiation between bacterial and viral infections.

Conclusions: In this study, the presence of innate immunity cytokines and chemokines were identified in saliva, allowing for a rapid identification and classification of infection as bacterial or viral. These data suggest that saliva can serve as a suitable, easily obtained source for rapid biomarker identification, which, when combined with standard of care, can lead to early diagnosis and improved prognosis for treatment of infected military personnel. Continued study of novel methodologies for rapid identification of biomarkers associated with microbial infections, may lead to improved treatment protocols, improved prognosis, and an overall decrease in the use of unnecessary antibiotics.

Introduction

Emerging respiratory disease agents, increased antibiotic resistance, and the reduction in the effectiveness of vaccines continues to increase the incidence of respiratory diseases in military personnel (1). Respiratory infections in military populations account for 25-30% of infectious disease hospitalization (2, 3). Because of the nature of the military environment, including group housing, stressful working conditions, and exposure to respiratory pathogens in endemic areas, military trainees and newly mobilized troops are at particularly high risk for acquisition of respiratory infections. Therefore, a rapid, sensitive, and field expedient methodology for early diagnosis and detection of respiratory infection is critically needed.

Current diagnosis and monitoring of infections often require invasive procedures by skilled technicians, including repeated blood draws, making analysis outside of a laboratory difficult. These challenges have made the use of saliva as a non-invasive, diagnostic tool increasingly popular. Sample gathering is less invasive than serum extraction and research has shown promise for saliva's use in detection of infection and genetic disease (4). Similar to serum, saliva contains electrolytes, proteins, nucleic acids, and cells of epithelial and immune origin. Because saliva is formed from filtration of blood, the inherent proteins and nucleic acids are similar to those found in serum (5, 6). This is particularly important as serum has long been used for the detection of various innate immune biomarkers, including cytokines and chemokines, for determining the immune system's response to pathogen exposure (7). Thus, saliva may serve as a novel, non-invasive diagnostic source for detection of innate immune biomarkers associated with respiratory infections.

Typically, when exposed to microorganisms (i.e., virus, bacteria, fungi, etc.), cells of the immune system secrete low molecular weight proteins called cytokines and chemokines, which induce an array of cellular responses including inflammation, production of antibodies, and engulfment of infected cells. When a bacterial infection occurs, the inflammatory response involves a number of cytokines and chemokines including: interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-17 (IL-17), Interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNFα), interleukin-8 (IL-8),

macrophage inflammatory protein-1-alpha (MIP-1α), macrophage inflammatory protein-1-beta (MIP-1β), monocyte chemoattractant protein -1 (MCP-1) eotaxin, RANTES, interferon gamma-induced protein-10 (IP-10), and growth factors such as basic fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor (VEGF) (8-10).

The humoral immune response is another major player against microbial invasion, which results in differentiation and activation of B-cells to produce antibodies against the bacterial proteins (11). This response includes cytokines such as IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, and IL-15, which play a role in B-cell differentiation and activation (11, 12). While similar immune pathways are activated in response to viral infections, the interferon (IFN) class I and III cytokines are the primary biomarkers, which are triggered through stimulation via viral antigens or viral dsRNA (13) to induce a number of cellular pathways such as proliferative inhibition and natural killer cell activation leading to an inhibition of viral replication and spread. These cytokines include interferon alpha/beta (IFN-α/β) (class I IFN), interleukin-28-alpha (IL-28α), interleukin-28-beta (IL-28β), and interleukin-29 (IL-29) (class III IFN) (14, 15).

Previous studies have demonstrated that a number of immune biomarkers are present in saliva and have utilized their expression patterns for diagnostic purposes in disease (16). In one study, researchers detected several different cytokines and chemokines in saliva by both reverse-transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) in healthy individuals vs. people with Sjogrens Syndrome (17). Additionally, Teles et al. (18) reported the detection of a broad array of cytokines and chemokines in whole saliva using a multiplex bead immunoassay in healthy individuals vs. patients with periodontitis.

The detection of immune and pathogen biomarkers in saliva has extended to diagnosing bacterial and viral infections including *Helicobacter pylori* and HIV (19, 20). Importantly, the expression pattern of a number of these immune biomarkers mirror that found in serum. Zhang et al. (21) demonstrated a positive correlation of TNFα, IL-6, and IL-8 in saliva vs. serum obtained from healthy subjects and people afflicted with a chronic inflammatory disease.

Additionally, several studies have defined expression patterns of cytokines and chemokines which differ between a bacterial and viral infection. Kimura et al. (22) found that the expression patterns of several cytokines and chemokines, including IL-1, IL-6, IFNα, and TNFα, differ when inducing the immune response in rabbits with lipopolysaccharide from bacteria and dsRNA mimicking nucleic acid. Serum levels of Procalcitonin, C-reactive protein, G-CSF, and human neutrophil lipocalin (HNL) have also been identified as possible markers for differentiating between viral and bacterial infections (23-25). Taken together, these studies suggest that saliva may serve as a matrix substrate used to differentiate between bacterial vs. viral infections using unique expression patterns of cytokines and chemokines. In this study, we evaluated the expression pattern of cytokines and chemokines in saliva and serum of healthy, bacterially infected, and virally infected individuals to identify biomarkers that could be utilized to differentiate between respiratory viral and bacterial infections via multiplex analysis.

Methods

Sample Collection and Processing

Samples of whole saliva and matched serum from healthy subjects (n=38) were purchased from Bioreclamations (Newbury, NY). Matched saliva and serum samples from bacterially or virally infected subjects (n=19 per group) were obtained from Discovery Life Sciences (Los Osos, CA) and Proteogenex (Culver City, CA). Clinical information was recorded for each subject (Tables 1-3). Subjects were labeled as "healthy" if no symptoms were observed before or during the study. Subjects defined as "virally infected" or "bacterially infected" displayed symptoms of an upper respiratory tract infection (Tables 2-3). Samples were aliquoted into microcentrifuge tubes and stored at -80°C until assaying.

Sample analysis

Multiplex protein analysis was performed using the magnetic human 27-plex cytokine group I panel and IFNα2 single-plex kit (Bio-rad, Hercules, CA) according to manufacturer instructions. Briefly, saliva samples were centrifuged at 12,000 x g at 4°C to remove cellular debris. Saliva supernatants were transferred to new centrifuge tubes and placed on ice. Standard curves were generated for each analyte using 4-fold serial

Bio-Rad *some places in print/online it is all CAPS.

seems the kit names would be capitalized

Did we check for Registered names, copyrights, Trademarks?

dilutions to generate 8 point curves ranging from 56,518 pg/ml to 0.24 pg/ml. Analysis was performed using magnetic beads in a 96-well flat bottom plate. Beads were washed twice with 300µl of Bio-plex wash buffer on the Biotek Ex405 magnetic plate washer followed by 50µl of standards or undiluted saliva samples added in technical duplicates and incubated for 1 hour at room temperature in the dark with shaking.

The beads were washed 3X in Bio-plex wash buffer and 25µl of the premixed detection antibodies were added to the beads and incubated for 30 minutes at room temperature in the dark with shaking. Beads were washed 3X with wash buffer and 50µl of streptavidin-PE was added followed by 10 minutes of incubation in the dark with shaking. Finally, beads were washed 3X with wash buffer and then resuspended in 125µl assay buffer and read on the Bio-plex 200 system using the low RPI setting. Data were analyzed using the Bio-plex manager software with 5PL curve fitting.

Statistical analysis

Significant differences between saliva and serum of healthy, bacterially, and virally infected subjects were determined using the student's t-test. A p-value of <0.05 was recorded as significant.

Results

Cytokine and chemokine levels of healthy subjects

To establish baseline cytokine/chemokine levels in saliva and serum, we measured the concentration of 28 analytes in a panel of 38 healthy subjects (Table 4). Of the 28 different cytokines/chemokines, only IL-1β, IL-1ra (receptor antagonist), IL-8, and VEGF had average baseline levels above 100 pg/ml in saliva. In serum, IL-8, IP-10, PDGF-BB (platelet-derived growth factor B-chain), and RANTES averaged above 100 pg/ml, demonstrating that most cytokines and chemokines in saliva and serum are normally present at low concentrations in healthy subjects.

Cytokine and chemokine levels of bacterially infected subjects

The saliva analysis of cytokine and chemokine expression levels in subjects with a clinically verified respiratory bacterial infection demonstrated 11 biomarkers that were significantly different from baseline levels of healthy subjects (Figure 1-2), including several which increased following infections and several which decreased. Increases or decreases in cytokine levels from baseline could be separated into two groups: $\geq$ 20 fold

changes and < 20 fold change vs. baseline. Cytokines/chemokines that were differentially regulated ≥ 20-fold included: IL-12 (49.7 vs. 0 pg/ml), IP-10 (19.7 vs. 1360 pg/ml, GM-CSF (65.4 vs. 0 pg/ml), and TNFα (2.47 vs. 116 pg/ml (Figure 1). Cytokines/chemokines that were found to be differentially regulated by an average of < 20 fold included: IL-1ra (38,224 vs. 9,297 pg/ml), IL-4 (0.58 vs. 2.2 pg/ml), IL-5 (0.24 vs. 1.26 pg/ml), IL-7 (2.09 vs. 11.05 pg/ml), IL-9 (3.17 vs. 0 pg/ml), VEGF (2,936 vs. 886 pg/ml), and IFNα2 (0 vs. 9.82 pg/ml (Figure 2). In serum, only IL-4 (1.38 vs. 6.66 pg/ml), IL-5 (2.09 vs. 5.91 pg/ml), and IP-10 (741 vs. 1,926 pg/ml) were found to be differentially regulated by an average of < 10-fold (Figure 3). When comparing saliva and serum biomarkers associated with bacterial respiratory infections, only IL-4, IL-5, and IP-10 were significantly different from healthy subjects in both saliva and serum (Figure 1-3).

Cytokine and Chemokine levels in virally infected subjects

The analysis of cytokines and chemokines in the saliva of healthy subjects compared to subjects with a confirmed respiratory viral infection revealed 17 cytokines/chemokines whose expression was significantly different (Figure 4-6). Cytokine/chemokine expression patterns in virally infected subjects could be separated into three distinct groups: ≥ 20 fold change, ≥ 10 fold change but < 20 fold, and < 10 fold change from baseline. The cytokines and chemokines that were differentially regulated by an average ≥ 20-fold included: IP-10 (19.7 vs. 1949 pg/ml), MIP-1α (0.16 vs. 8.04 pg/ml), MIP-1β (0.48 vs. 17.8 pg/ml), GM-CSF (65.5 vs. 2.34 pg/ml), TNFα (2.47 vs. 72 pg/ml), and IFNα2 (0 vs. 56 pg/ml) (Figure 4). Cytokines/Chemokines that were found to be differentially regulated by an average of ≥ 10 but < 20 fold included: IL-5 (0.24 vs. 3.85 pg/ml), IL-7 (2.09 vs. 23.2 pg/ml), IL-13 (0.39 vs. 6.31 pg/ml), VEGF (2936 vs. 279 pg/ml), and eotaxin (3.46 vs. 39.5 pg/ml) (Figure 5). Cytokines/chemokines that were differentially regulated by an average of < 10 included: IL-1ra (38,224 vs. 4,756 pg/ml), IL-4 (0.58 vs. 2 pg/ml), IL-8 (1056 vs. 255 pg/ml), IL-9 (3.17 vs. 13.5 pg/ml), IL-12 (49.7 vs. 113 pg/ml), and PDGF-BB (6.21 vs. 50 pg/ml) (Figure 6). The serum of virally infected subjects demonstrated 12 cytokines/chemokines that were significantly different from healthy subjects (Figure 7, 8). The overall changes in expression pattern were not as robust as those observed in

saliva, but could be separated into two groups, ≥ 10 fold or < 10 fold change from baseline levels. Cytokines/ chemokines whose expression differed by an average of ≥ 10 fold included: MCP-1 (0.57 vs. 25.76 pg/ml), G-CSF (53.7 vs. 1,360 pg/ml), VEGF (0 vs. 51.8 pg/ml), and eotaxin (5.1 vs. 75 pg/ml) (Figure 7), while those with average changes of < 10-fold included: IL-1β (1.41 vs. 2.69 pg/ml), IL-4 (1.38 vs. 3.67 pg/ml), IL-6 (7.03 vs. 22 pg/ml), IL-9 (2.33 vs. 16.1 pg/ml), IP-10 (741 vs. 2,520pg/ml), MIP-1β (60.5 vs. 117 pg/ml), TNFα (14 vs. 64 pg/ml), and IFN-γ (47 vs. 84 pg/ml) (Figure 8). In analyzing cytokines/chemokines for identification of an active viral infection in both saliva and serum, IL-4, IL-9, eotaxin, IP-10, MIP-1β, and TNFα were significantly differentially regulated in virally infected subjects as compared to healthy individuals (Figure 4-8). Interestingly, VEGF was up-regulated in serum and down-regulated in saliva (Figure 5, 7).

Cytokines and Chemokines differentially expressed in respiratory bacterial vs. viral infections

A number of cytokines/chemokines were identified, which allowed for detection and discrimination between viral vs. bacterial infections, including IL-8, IL-9, IL-12, IL-13, eotaxin, and IFNα2 (Table 5). Notably, in healthy subjects, IL-8 was detected in all subjects (100%) with an average of 1,056 pg/ml (36-6,656 pg/ml). In virally infected subjects, IL-8 was detected in all subjects (100%) with significant down-regulation as compared to healthy subjects, showing an average of 255 pg/ml (48-1,190 pg/ml). In contrast, although IL-8 was detected in all bacterially infected subjects (100%), it was not significantly up-regulated as compared to healthy subjects displaying an average of 2,498 pg/ml (79-13,931 pg/ml). Thus, IL-8 demonstrated a significant difference in expression between viral vs. bacterial infections. IL-9 also proved to be differentially expressed between bacterial and viral infections. Specifically, in healthy subjects, IL-9 was detectable in 8 of the 38 subjects tested (21%) with an average of 3.2 pg/ml (2.3-48 pg/ml). However, IL-9 was significantly up-regulated in all virally infected subjects with an average of 13.5 pg/ml (1-42.6 pg/ml). In contrast, IL-9 was below the level of detection in all 19 bacterially infected subjects examined (0%).

IL-12 also allowed discrimination between viral and bacterial infections. In healthy subjects, IL-12 was detected in 30 of the 38 subjects tested (78%) with an

average of 50 pg/ml (9.5-151 pg/ml). In virally infected subjects, however, IL-12 was detected in all subjects (100%) and significantly up-regulated with an average of 113 pg/ml (45-259 pg/ml). In contrast, IL-12 expression in bacterially infected subjects was below the level of detection in all subjects (0%). Another marker we identified for distinguishing between viral and bacterial infections was IL-13. IL-13 was seen in 13 of the 38 healthy subjects examined (34%) with an average of 0.39 pg/ml (0.1-4.3 pg/ml). When tested in virally infected subjects, IL-13 was significantly up-regulated and observed in 16 of the 19 subjects (85%) with an average of 6.3 pg/ml (1.5-20.7 pg/ml). In bacterially infected subjects, however, IL-13 was seen in 9 of the 19 subjects (47%) with an average of 1.1 pg/ml (0.5-5.2 pg/ml), and showed no significant difference when compared to healthy subjects.

Eotaxin was also differentially expressed between viral and bacterial infections. In healthy subjects, eotaxin was detected in 3 of the 38 subjects tested (8%) with an average of 3.5 pg/ml (11-93 pg/ml). In virally infected subjects, eotaxin was significantly up-regulated and observed in 95% of the subjects with an average of 40 pg/ml (3.1-126 pg/ml). When examined in bacterially infected subjects, eotaxin was detectable in only 1 of the 19 subjects (5%), with a level of 24 pg/ml, and no significant difference was observed between healthy and bacterially infected subjects. Lastly, IFNα2 was differentially expressed between bacterial and viral infections. In healthy subjects, IFNα2 was below the level of detection in all subjects tested (0%). Virally infected subjects demonstrated detectable levels of IFNα2 in 18 of the 19 subjects tested (95%), displaying significant up-regulation with an average of 56 pg/ml (1.6-255 pg/ml). In bacterially infected subjects, IFNα2 was detected in 7 of the 18 subjects examined (39%) showing significant up-regulation with an average of 9.8 pg/ml (8.8-60 pg/ml). However, IFNα2 in virally infected subjects was still significantly higher than those in bacterially infected subjects.

Serum samples only revealed 3 markers that allowed discrimination between bacterial and viral infections, including IL-4, IL-5, and VEGF. In healthy subjects, IL-4 was detected in 21 of the 38 subjects tested (55%) with an average of 1.38 pg/ml (0.37-4.6 pg/ml). In virally infected subjects, IL-4 was detected in all subjects (100%) and significantly up-regulated with an average of 3.67 pg/ml (1.3-7.3 pg/ml). In bacterially

infected subjects, IL-4 was also detected in all subjects (100%) and was expressed at significantly higher levels than both healthy and virally infected subjects with an average of 6.6 pg/ml (1.3-15 pg/ml). In healthy subjects, IL-5 was detected in 19 of the 38 subjects tested (50%) with an average of 2.1 pg/ml (1.6-11.8 pg/ml). All virally infected subjects expressed IL-5 (100%) with an average of 1.9 pg/ml (0.25-7 pg/ml). Although there was no statistical significance between healthy and virally infected subjects, all bacterially infected subjects expressed IL-5 (100%) with an average of 5.9 pg/ml (1.2-18 pg/ml). Statistical comparison revealed a significant difference between healthy and bacterially infected subjects, as well as bacterially infected and virally infected subjects. VEGF was below the level of detection in the serum of all healthy subjects examined (0%). In virally infected subjects, VEGF was significantly up-regulated and detectable in 10 of the 19 subjects tested (52%) with an average of 52 pg/ml (11.4-265 pg/ml). In bacterially infected subjects however, VEGF was below the level of detection in all subjects (0%).

Discussion

Biomarkers play an increasingly important role in diagnostics for detection of infection and disease. Because the clinical symptoms of viral and bacterial respiratory infections are very similar, it is difficult for doctors to distinguish between these types of infections, often leading to misdiagnosis and inappropriate treatment. Previous studies have focused on using serum as the sample source for detection of biomarkers in infection and disease; however, saliva is attracting more attention due to its non-invasive collection and ease of processing (26).

Our data identified a total of 17 cytokine/chemokine biomarkers that were differentially regulated in saliva between healthy and virally infected subjects and 12 cytokine/chemokine biomarkers in serum. In healthy vs. bacterially infected subjects, a total of 11 biomarkers in saliva and 3 in serum were differentially regulated. From the set of biomarkers that we identified to be differentially regulated between healthy subjects and subjects with a confirmed bacterial or viral infection, IL-8, IL-9, IL-12, IL-13, eotaxin, and IFNα2 may allow the differentiation between viral and bacterial respiratory infections in saliva, while IL-4, IL-5, and VEGF could be used in serum.

Previous studies have found a number of biomarkers that are differentially regulated in bacterial and/or viral infections as compared to healthy subjects, although there is variability from study to study. Chalupa et al. (27) tested for 7 different cytokines and chemokines in serum, as well as a few acute phase proteins, white blood cells counts, and surface expression of several T-cell receptors. In their study, only procalcitonin in serum could differentiate between viral and bacterial infections, although their biomarker panel did not include IL-4, IL-5, or VEGF. Additionally, they used serum only and examined a range of different types of bacterial and viral infections rather than focusing solely on respiratory infections.

Biomarker G-CSF has also been found to differentiate between bacterial and viral infections in serum. Indeed, an early study found that serum levels of G-CSF were rapidly increased in subjects with acute bacterial infections, but not with viral infections or Mycoplasma pneumonia infections (23). Our study found that serum G-CSF was increased in subjects with a respiratory viral or bacterial infection as compared to healthy subjects, but significant differences were only observed in subjects with an ongoing viral infection. This discrepancy between studies could be dependent on the kinetics of inflammation, associated with the timing of infection prior to seeking medical attention.

Several studies have found that IP-10 could also be used as an early diagnostic biomarker for bacterial infections (28). Li et al. (29) found that in HSG cells, which originate from human submandibular ducts, IP-10 was expressed in response to polyinosinic: polycytidylic acid (Poly:IC), an artificial mimic of the dsRNA genomes of viruses, which induces an immune response similar to real viral infections. Similar results were found by Jiang and others (30), where IP-10 was markedly increased in the blood of subjects infected with severe acute respiratory syndrome (SARS) (30). In our study, we found that IP-10 in both serum and saliva was significantly up-regulated in bacterially and virally infected subjects as compared to healthy subjects. However, there was no significant difference in IP-10 expression levels in bacterially vs. virally infected subjects suggesting that while IP-10 may not be suitable for distinguishing between viral and bacterial infections, IP-10 may serve as a reliable biomarker for detecting an infection in general.

A recent study examining the levels of cytokines and chemokines in serum found that IL-1ra, IL-2, IL-6, and TNFα were significantly higher in the serum of bacterially infected subjects than virally infected or healthy subjects, while MCP-1 and MIP-1β were higher in bacterially infected individuals as compared to healthy individuals (31). In our study, IL-1ra, IL-6, and TNFα were increased in the serum of bacterially and virally infected subjects as compared to healthy subjects, but none reached significance. Likewise, IL-2 was increased in the serum of bacterially infected subjects as compared to healthy subjects but without showing a significant difference. Interestingly, while both MCP-1 and MIP-1β were significantly increased in the serum of virally infected subjects as compared to healthy subjects, no significant difference was detected between healthy and bacterially infected subjects.

In saliva, IL-1ra was significantly down-regulated in both bacterially and virally infected subjects as compared to healthy subjects. TNFα on the other hand, was significantly up-regulated in the saliva of both bacterially and virally infected individuals as compared to healthy subjects. MIP-1β though, was only significantly up-regulated in virally infected individuals but not bacterially infected individuals. Lastly, MCP-1 expression levels were higher in the saliva of bacterially infected subjects as compared to virally infected subjects, but these differences did not reach significance.

Other biomarkers that were differentially regulated between viral and bacterial infections in our report include IFNα2, IL-12, and eotaxin. The saliva of virally infected subjects contained significantly higher levels of IFNα2 compared to bacterially infected subjects. This is not surprising given the fact that IFNα is highly expressed in viral infections and typically, is known to be the hallmark of an anti-viral immune response (32). Type I interferons, such as IFNα, are also known to up-regulate IL-9 through the expression of IL-21 (33). Concurrent with this, we found IL-9 to be significantly up-regulated in the saliva of virally infected subjects, but not bacterially infected subjects.

IL-12 has been shown to be up-regulated in bacterial and viral infections, such as *Staphylococcus aureus* and Influenza virus, and plays a key role in cell-mediated immunity, particularly against intracellular pathogens such as viruses (34-36). Our results demonstrate IL-12 to be down-regulated in the saliva of bacterially infected subjects as compared to healthy subjects, but up-regulated in virally infected subjects.

IL-8 was found to be up-regulated in the saliva of bacterially infected subjects, but down-regulated in virally infected subjects. This important chemokine is responsible for activating neutrophils in the inflammatory response and studies have found a number of infections and diseases, including cancer, that up-regulate IL-8 suggesting usefulness as a potential biomarker (37).

In saliva, eotaxin was identified as a positive candidate for bacterial and viral infection differentiation. Eotaxin is a chemokine responsible for recruiting eosinophils, with the airway epithelial cells serving as a major source of eotaxin expression. Previous reports have shown that respiratory viral infections, including rhinovirus and influenza virus can induce expression of eotaxin in nasal and bronchial epithelial cells (38, 39). In agreement with this, our data revealed that eotaxin was significantly up-regulated in the saliva of virally infected subjects, but not bacterially infected subjects. The lack of eotaxin stimulation by bacteria is supported by Issa et al. (40) who demonstrated that *E. coli* and *S. aureus* tend to inhibit eotaxin release in airway smooth epithelial cells.

In terms of comparing saliva to serum, six cytokine/chemokine biomarkers were significantly up-regulated in both saliva and serum when comparing healthy subjects to virally infected subjects while one marker, VEGF, was down-regulated in the saliva of virally infected subjects and up-regulated in serum. When comparing healthy subjects to bacterially infected subjects, only 3 markers were up-regulated in both saliva and serum. The most likely explanation for identification of more biomarkers in saliva as compared to serum for both types of infection may be that all subjects included in our analysis were confirmed to have upper respiratory infections, thus potentially allowing for an enhancement of a local immune response observed in saliva, but lacking systemically. The inability to identify a common set of cytokine/chemokine biomarkers in both saliva and serum suggests a weak correlation between saliva and serum biomarkers, at least for respiratory infections.

While our results indicate the suitability of several biomarkers for the differentiation between viral and bacterial infections, there are noteworthy limitations of this study. First, a larger subject sample size should be utilized to confirm the presence and expressional differences in saliva and serum biomarkers. Additionally, as samples

were not obtained from a controlled clinical study, there was no opportunity to evaluate the kinetic expression patterns on the biomarkers, which could be instrumental in early identification of viral or bacterial infections.

Lastly, a broader spectrum of microbial infections should be included in future studies. While our study did include a variety of bacterial species for respiratory infections, our virally infected subjects were limited to influenza only. Influenza is a seasonal virus and thus provided a good source for obtaining samples. However, different viruses can have distinct effects on the expression of inflammatory cytokines, and should be evaluated for both common and different cytokine/chemokine biomarker profiles.

In conclusion, our data has identified several cytokine/chemokine biomarkers in saliva and serum that could be used to assist clinicians in correctly diagnosing a viral or bacterial infection. Importantly, our results also reveal that saliva can serve as a viable sample source in differentiating between respiratory viral and bacterial infections and is compatible with multiplex bead analysis. Future studies with larger sample sizes and a broader range of respiratory infections may provide confirmation of specific biomarkers and significant advancements in diagnostic capability and delivery of early, targeted treatments.

Military Significance

A method for early diagnostic detection of infections is critically important for military members. Warfighters run the risk of not only becoming exposed to biological agents, but also have an increased risk of spreading the infection due to constrained quarters, especially on naval ships at sea, or during training periods. Warfighters have a greater susceptibility to acquiring infections due to extended periods of physical exertion that can lead to exhaustion and ultimately, compromise the immune response. Thus, it is important to have a method of rapid detection that can aid in accurate diagnoses of the type of infection that is present. The ability to utilize saliva as a sample source for differentiating between viral and bacterial respiratory infections may allow military field physicians to quickly and accurately diagnose infections without invasive procedures. Infections could then be swiftly contained and treated, thereby allowing for improved prognosis and treatment regimens for our war fighters.

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Tables

Table 1. Subject Information for Healthy Subjects

Subject #	Age	Gender	Ethnicity	Specimen type
1	50	male	white	Saliva/serum
2	48	male	black	Saliva/serum
3	30	female	black	Saliva/serum
4	53	male	black	Saliva/serum
5	42	male	black	Saliva/serum
6	33	male	black	Saliva/serum
7	44	male	black	Saliva/serum
8	19	male	black	Saliva/serum
9	28	male	black	Saliva/serum
10	42	male	black	Saliva/serum
11	38	male	white	Saliva/serum
12	31	male	black	Saliva/serum
13	28	male	white	Saliva/serum
14	48	male	white	Saliva/serum
15	45	male	black	Saliva/serum
16	37	male	black	Saliva/serum
17	25	male	hispanic	Saliva/serum
18	52	male	black	Saliva/serum
19	46	male	black	Saliva/serum
20	33	female	black	Saliva/serum
21	39	male	black	Saliva/serum
22	46	male	hispanic	Saliva/serum

Table 1 Continued. Subject Information for Healthy Subjects

Subject #	Age	Gender	Ethnicity	Specimen type
23	53	male	black	Saliva/serum
24	56	male	black	Saliva/serum
25	27	male	black	Saliva/serum
26	24	male	hispanic	Saliva/serum
27	24	female	black	Saliva/serum
28	19	male	black	Saliva/serum
39	20	male	black	Saliva/serum
30	47	female	black	Saliva/serum
31	35	female	black	Saliva/serum
32	41	male	white	Saliva/serum
33	30	female	white	Saliva/serum
34	43	male	black	Saliva/serum
35	34	male	black	Saliva/serum
36	49	male	black	Saliva/serum
37	25	male	hispanic	Saliva/serum
38	27	female	white	Saliva/serum

Table 2. Subject Information for Bacterially Infected Subjects

Subject	Age	Gender	Ethnicity	Specimen type	Symptoms	Diagnostics	Diagnosis
1	24	female	White	Saliva/serum	Cough, fatigue, fever, sore throat	Vitek 2 compact	*Streptococcus pneumoniae*
2	58	male	white	Saliva/serum	Cough, fatigue, fever, sore throat	Vitek 2 compact	*Streptococcus pneumoniae*
3	52	male	white	Saliva/serum	Cough, fatigue, fever, nasal congestion, sneezing,	Vitek 2 compact	*Haemophilus Influenzae*
4	51	female	white	Saliva/serum	Cough, fever, muscle ache, nausea, fatigue	Vitek 2 compact	*Klebsiella pneumoniae*
5	55	male	white	Saliva/serum	Cough, fever	Vitek 2 compact	*Staphylococccus aureus*
6	27	male	white	Saliva/serum	Cough, fatigue, fever, sneezing	Vitek 2 compact	*Staphylococccus aureus*
7	39	female	white	Saliva/serum	Cough, fever, fatigue, sore throat	Vitek 2 compact	*Streptococcus pneumoniae*
8	44	female	white	Saliva/serum	Cough, fever, muscle aches, nausea	Vitek 2 compact	*Klebsiella pneumoniae*
9	50	female	white	Saliva/serum	Not available	Not available	*Haemophilus influenzae, Serratia marcescens, enterococcus aerogenes*
10	60	female	white	Saliva/serum	Not available	Not available	Rare Beta hemolytic GBS
11	46	male	white	Saliva/serum	Not available	Not available	*Pseudomonas aeruginosa*
12	78	male	white	Saliva/serum	Not available	Not available	*Acinetobacter lwoffii, Leclercia adecarboxylata*
13	87	female	white	Saliva/serum	Not available	Not available	*MRSA*
14	15	male	black	Saliva/serum	Not available	Not available	*Proteus mirabilis, Providencia stuartii*
15	71	male	white	Saliva/serum	Not available	Chest x-ray	Bacterial pneumonia
16	67	female	white	Saliva/serum	Not available	Chest x-ray	Bacterial pneumonia
17	69	male	white	Saliva/serum	Not available	Chest x-ray	Bacterial pneumonia
18	70	male	White	Saliva/serum	Not available	Chest x-ray	Bacterial pneumonia
19	65	male	white	Saliva/serum	Not available	Chest x-ray	Bacterial pneumonia

Table 3. Subject Information for Virally Infected Subjects

Subject	Age	Gender	Ethnicity	Specimen type	Symptoms	Diagnostics	Diagnosis
1	47	Male	White	Saliva/serum	Cough, fatigue, fever, sore throat, sneezing	SD bioline	Influenza A
2	56	female	white	Saliva/serum	Cough, fatigue, fever, sore throat, sneezing	SD bioline	Influenza A
3	45	male	white	Saliva/serum	Cough, fever, nasal congestion, sneezing, sore throat	SD bioline	Influenza A
4	52	female	white	Saliva/serum	Cough, fever, sneezing, sore throat, fatigue	SD bioline	Influenza A
5	50	male	white	Saliva/serum	Cough, fever, sneezing, congestion, sore throat	SD bioline	Influenza A
6	57	female	white	Saliva/serum	Cough, fatigue, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
7	39	male	white	Saliva/serum	Cough, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
8	37	male	white	Saliva/serum	Cough, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
9	54	male	white	Saliva/serum	Cough, fever, congestion, sneezing	SD bioline	Influenza A
10	45	male	white	Saliva/serum	Cough, fever, fatigue, congestion, sneezing	SD bioline	Influenza A
11	50	male	white	Saliva/serum	Cough, fatigue, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
12	53	male	white	Saliva/serum	Cough, fatigue, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
13	59	male	white	Saliva/serum	Cough, fatigue, fever, congestion, aches	SD bioline	Influenza A
14	59	female	white	Saliva/serum	Cough, fatigue, fever, congestion, sneezing, sore throat	SD bioline	Influenza A
15	27	male	white	Saliva/serum	Cough, running nose, chill	N/A	Suspected viral infection
16	49	female	white	Saliva/serum	Cough, sore throat	N/A	Suspected viral infection
17	49	female	white	Saliva/serum	Cough, headache, chill, fever	N/A	Suspected viral infection
18	29	male	White	Saliva/serum	Cough, sore throat, fever	N/A	Suspected viral infection
19	40	male	white	Saliva/serum	Cough, sore throat, fever, chill	N/A	Suspected Viral infection

Table 4. Cytokine and Chemokine Levels in the Saliva and Serum of Healthy Subjects

Cytokine/Chemokine	Saliva Average (pg/ml)	Saliva Range (pg/ml)	Serum average (pg/ml)	Serum range (pg/ml)
IL-1β	908	0.83-17997	1.41	0-10.49
IL-1ra	38224	978-217256	68	0-1599
IL-2	5.73	0-90.86	0	0
IL-4	0.58	0-3.41	1.38	0-4.64
IL-5	0.24	0-1.41	2.09	0-11.79
IL-6	8.03	0-111.77	7	0-124.18
IL-7	2.09	0-9.77	4.78	0-64.19
IL-8	1056	36.08-6656	131	0-1795
IL-9	3.16	0-47.96	2.32	0-88.41
IL-10	28	0-66.99	4.5	0-86.34
IL-12	49.69	0-150.79	13.65	0-431.62
IL-13	0.39	0-4.28	22.03	0-765.06
IL-15	9.075	0-183.53	0	0
IL-17	6.84	0-211	24.8	0-723.88
Eotaxin	3.46	0-93.38	5.09	0-193.68
FGF basic	17.51	0-263.67	19.84	0-474.7
IP-10	19.7	0-211.06	741	235.41-2631
G-CSF	56.6	2.81-255.53	53.7	0-322.65
GM-CSF	65.45	0-209.83	0	0
IFN-γ	35.64	0-433.62	46.95	0-156.28
MCP-1	86.39	0-564.63	0.57	0-8.8
MIP-1α	0.16	0-1.95	20.5	0-528.45
MIP-1β	0.48	0-7.04	60.47	0-447.5
RANTES	2.7	0-62.68	8870.9	60.61-20000
TNF-α	2.47	0-30.29	14	0-216.84
VEGF	2936	33.79-12089	0	0
PDGF-BB	6.2	0-106.53	2438	0-12221
IFNα2	0	0	0	0

Table 5. Cytokines/Chemokines Differentiation between Viral and Bacterial Infections

	Saliva (pg/ml)			Serum (pg/ml)			
Cytokine/Chemokine	**Healthy**	**Bacterial**	**Viral**	**Cytokine/Chemokine**	**Healthy**	**Bacterial**	**Viral**
IL-8	1056 (36-6656)	2498 (79-13931)	255 (48-1190)	**IL-4**	1.38 (0.37-4.6)	6.6 (1.3-15)	3.67 (1.3-7.3)
IL-9	3.2 (2.3-48)	0	13.5 (1-42.6)	**IL-5**	2.1 (1.6-11.8)	5.9 (1.2-18)	1.9 (0.25-7)
IL-12	50 (9.5-151)	0	113 (45-259)	**VEGF**	0	0	52 (11.4-265)
IL-13	0.39 (0.1-4.3)	1.1 (0.5-5.2)	6.3 (1.5-20.7)				
Eotaxin	3.5 (11-93)	1.3 (24)	40 (3.1-126)				
IFNα2	0	9.8 (8.8-60)	56 (1.6-255)				

Figures

Figure 1.

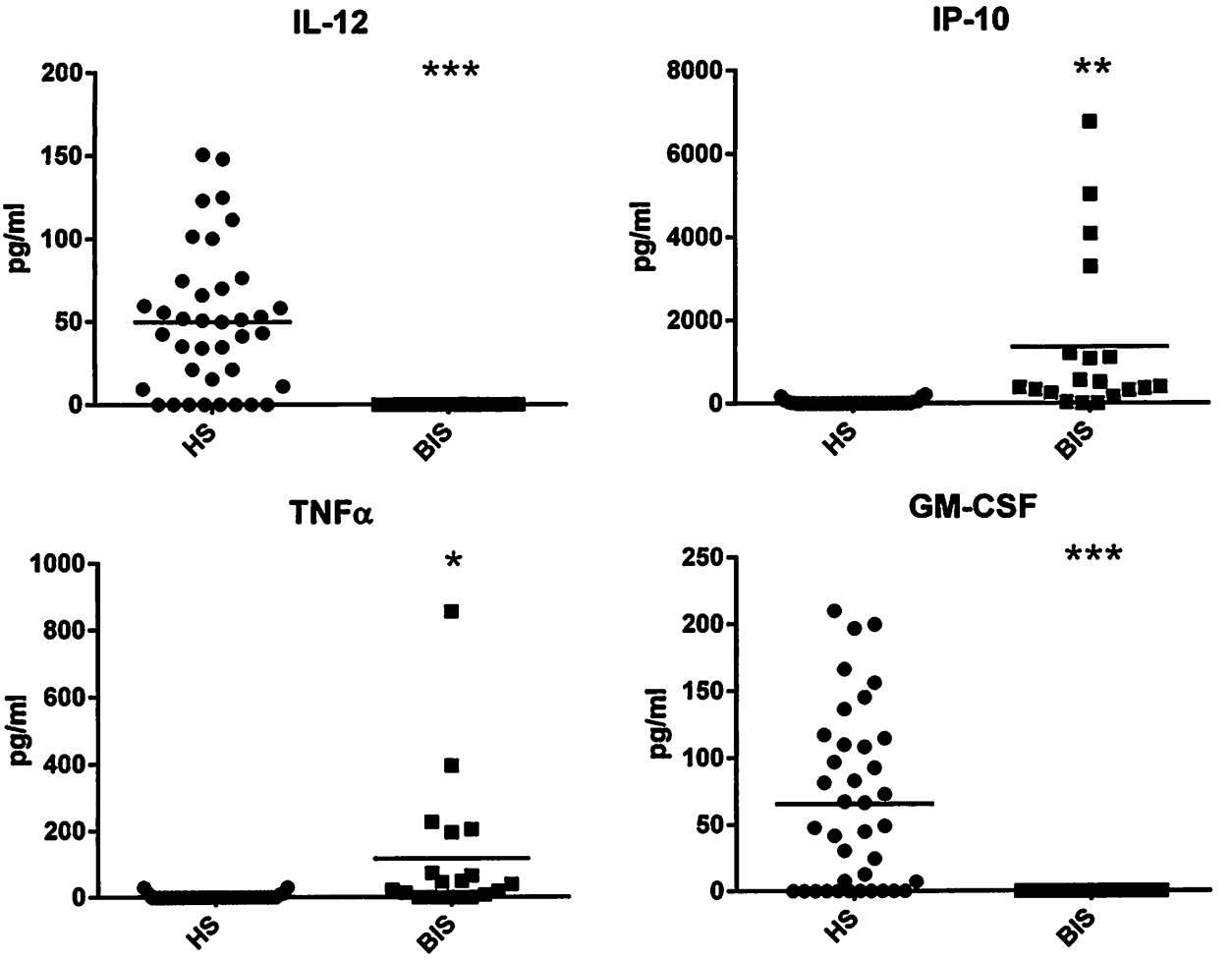

Figure 1. Cytokines and chemokines differentially regulated by an average of ≥ 20 fold between healthy and bacterially infected subject saliva. Saliva from healthy or bacterially infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$;**, $p \leq 0.01$; *, $p \leq 0.05$). HS-healthy saliva, BIS-bacterially infected saliva.

Figure 2.

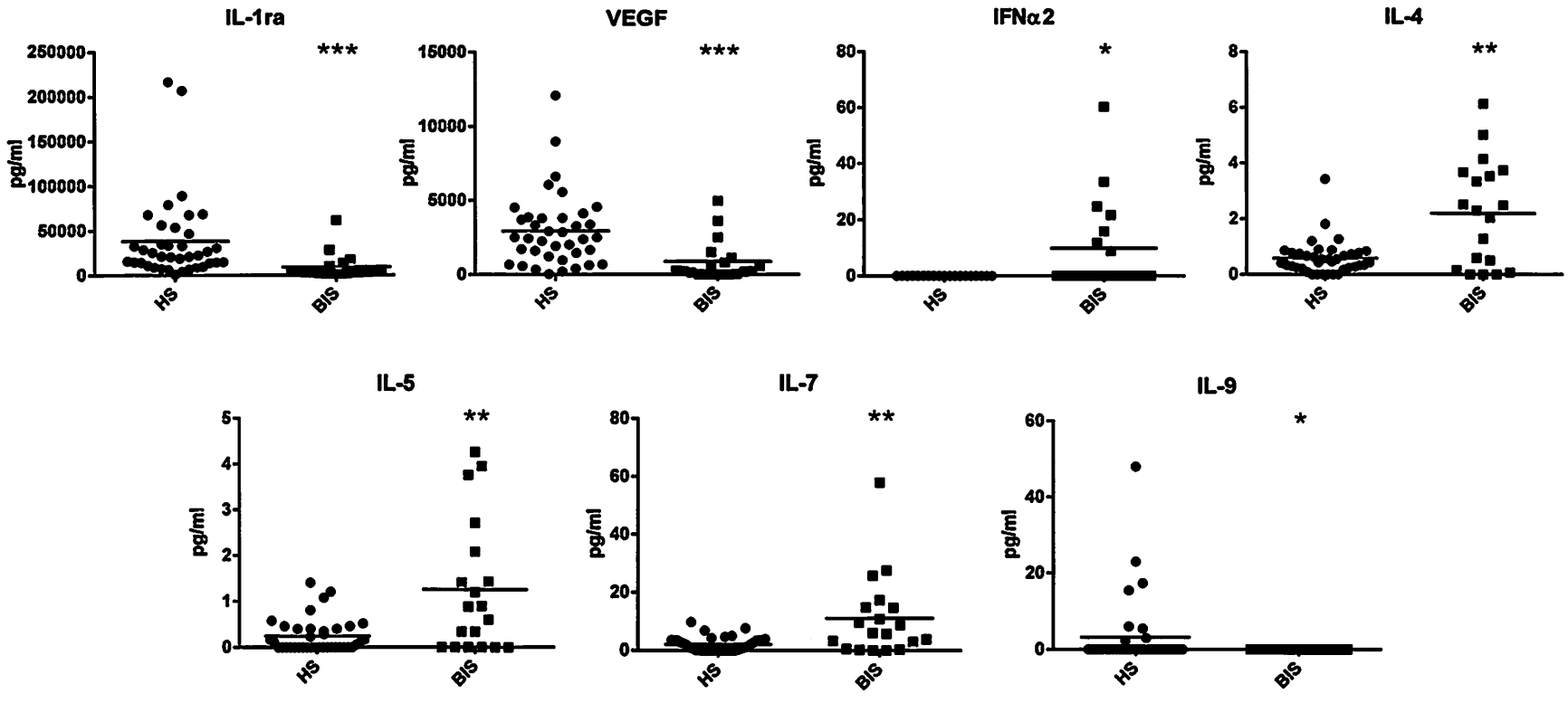

Figure 2. Cytokines and chemokines differentially regulated by an average of < 20 fold between healthy and bacterially infected subject saliva. Saliva from healthy or bacterially infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$). HS-healthy saliva, BIS-bacterially infected saliva.

Figure 3.

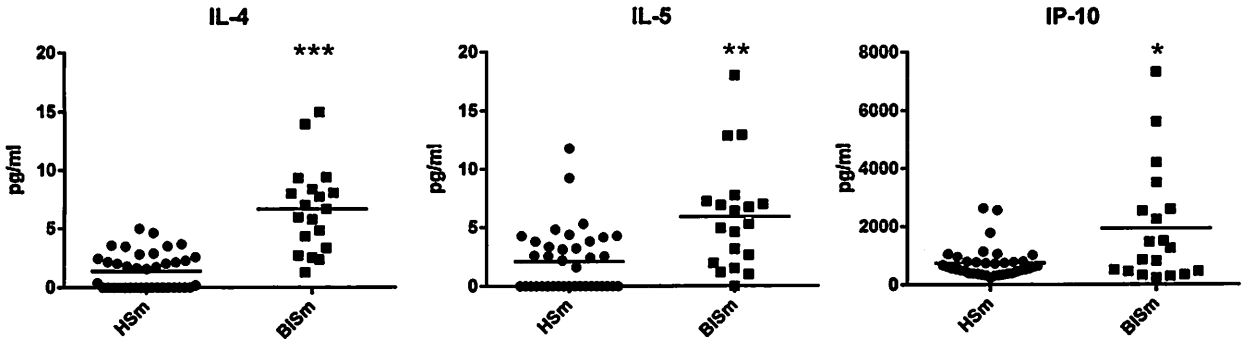

Figure 3. Cytokines and chemokines differentially regulated by an average of ≤ 10 fold between healthy and bacterially infected subject serum. Serum from healthy or bacterially infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$). HSm-healthy serum, BISm-bacterially infected serum.

Figure 4.

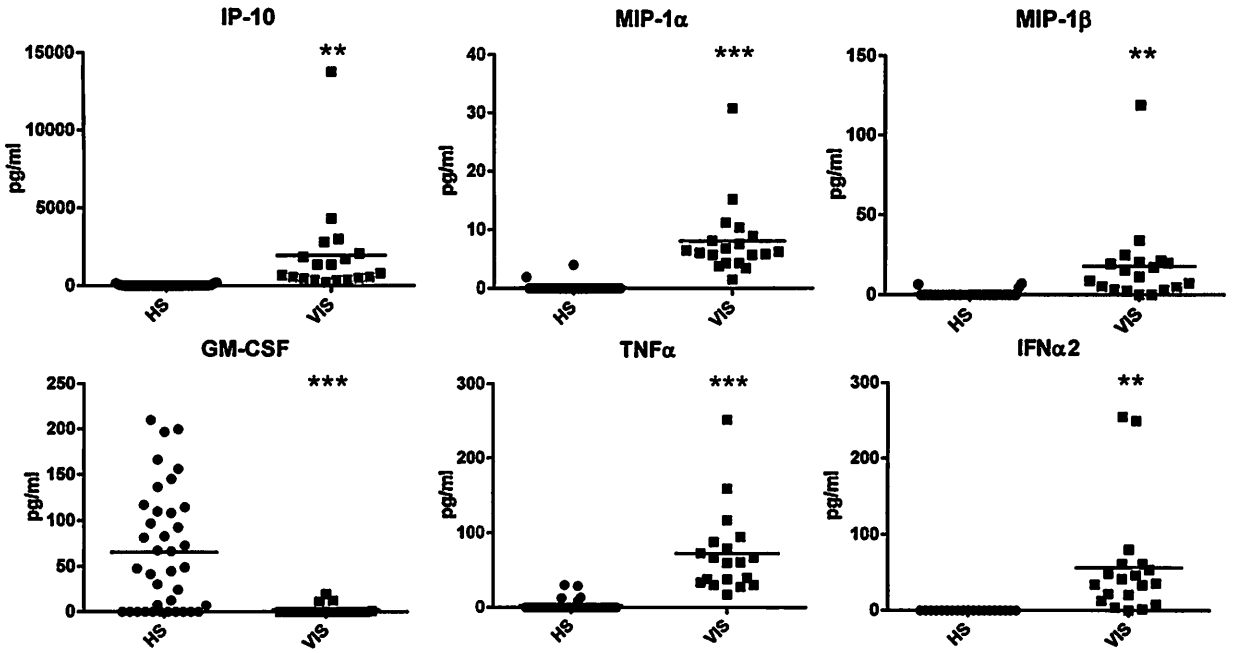

Figure 4. Cytokines and chemokines differentially regulated by an average of ≥ 20 fold between healthy and virally infected subject saliva. Saliva from healthy or virally infected subjects was analyzed by multiplex analysis for cytokine/ chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$). HS-healthy saliva, VIS-virally infected saliva.

Figure 5.

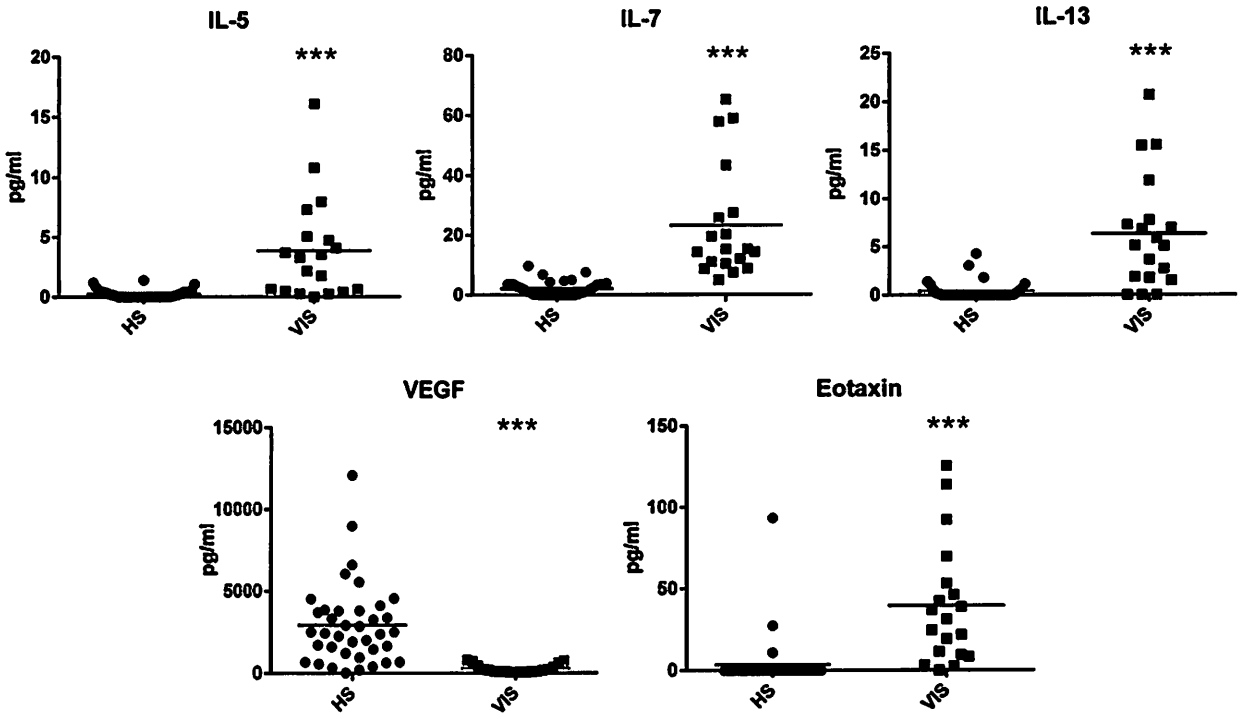

Figure 5. Cytokines and chemokines differentially regulated by an average of ≥ 10 fold but < 20 fold between healthy and virally infected subject saliva. Saliva from healthy or virally infected subjects was analyzed by multiplex analysis for cytokine/ chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$). HS-healthy saliva, VIS-virally infected saliva

Figure 6.

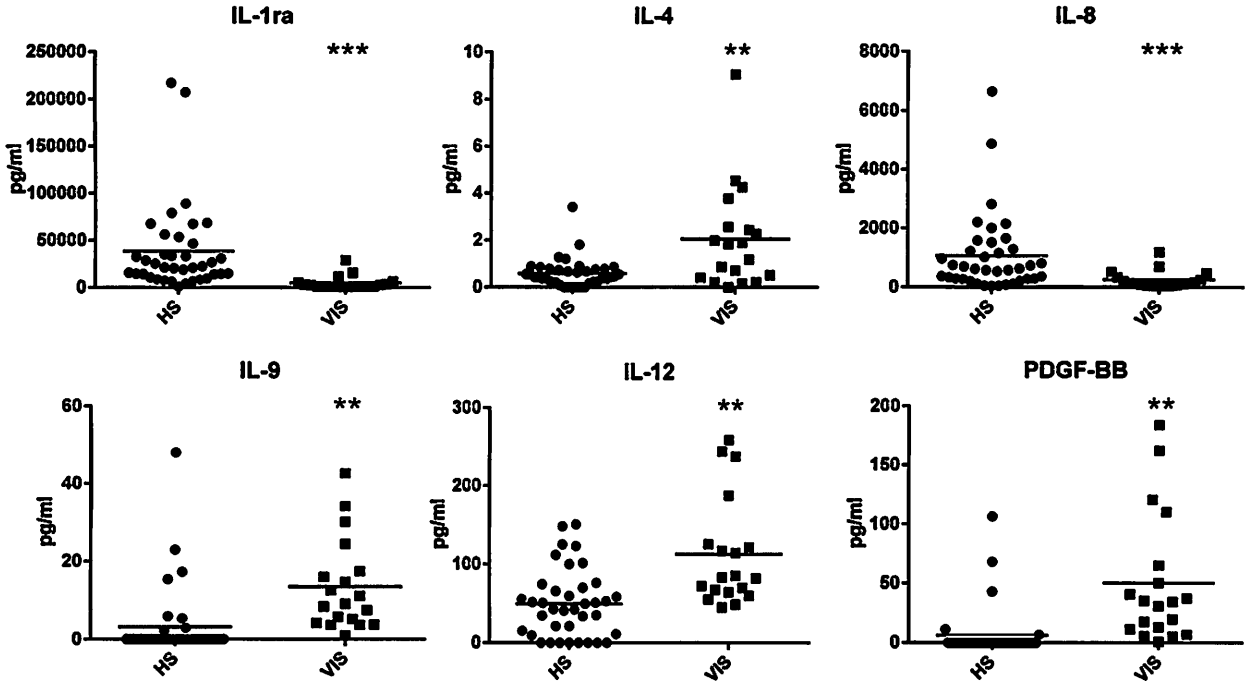

Figure 6. Cytokines and chemokines differentially regulated by an average of < 10 fold between healthy and virally infected subject saliva. Saliva from healthy or virally infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$). HS-healthy saliva, VIS-virally infected saliva.

Figure 7.

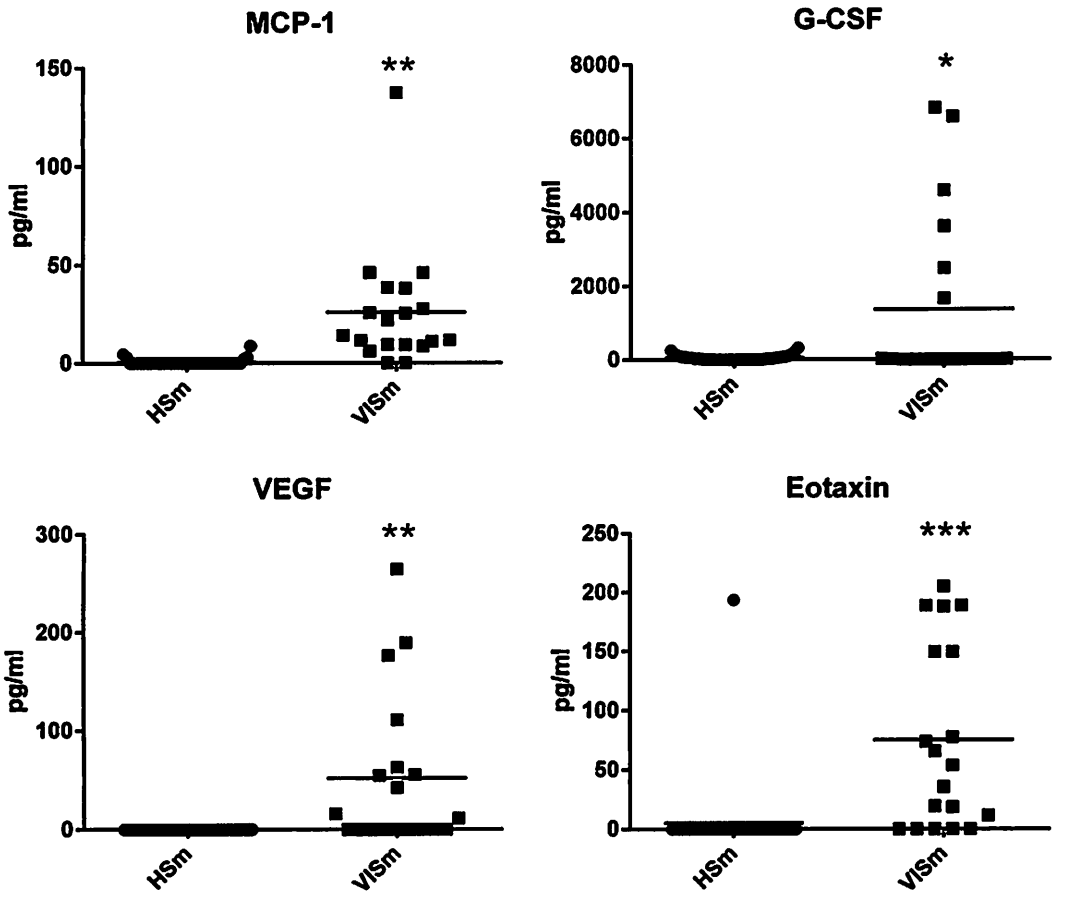

Figure 7. Cytokines and chemokines differentially regulated by an average of ≥ 10 fold between healthy and virally infected subject serum. Serum from healthy or virally infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$). HSm-healthy serum, VISm-virally infected serum.

Figure 8.

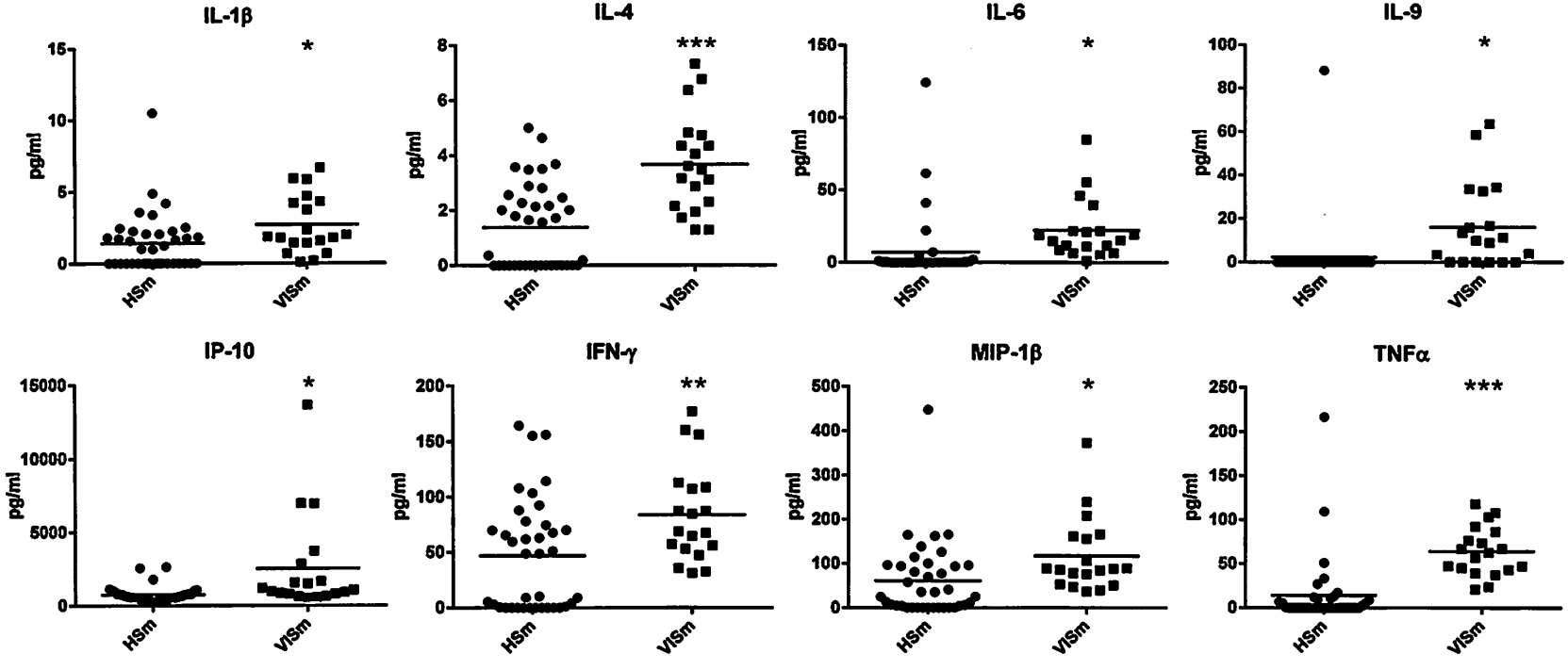

Figure 8. Cytokines and chemokines differentially regulated by an average of < 10 fold between healthy and virally infected subject serum. Serum from healthy or virally infected subjects was analyzed by multiplex analysis for cytokine/chemokine expression. Each dot represents a single subject sample. Average concentrations were determined for each group (cross bar). Statistical significance was determined by the student's t-test (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$) HSm-healthy serum, VISm-virally infected serum

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14. ABSTRACT

Military group housing, training facilities, and operational theatres, combined with high stress, presents unique environments for dissemination and propagation of transmitting bacterial and viral infections. While often associated with mild illness, severe disease may occur with significant morbidity, leading to a detrimental impact on training schedules and operational readiness. Current diagnosis and monitoring of infections require invasive procedures by skilled technicians, including repeated blood draws, making it difficult for in-theatre care. Therefore, there remains a critical need for a rapid, sensitive assay for detection and diagnosis of microbial infections in our warfighters, both in garrison and in theatre. The objective of this study was to explore the presence of innate immune biomarkers in saliva associated with bacterial and viral respiratory infections, as compared to markers present in serum samples. A panel of 28 cytokines and chemokines in saliva and serum obtained from 38 healthy subjects and 19 bacterially infected or virally infected individuals were analyzed via bio-plex analysis. A unique set of innate immune biomarkers, including: IL-8, IL-9, IL-12, IL-13, eotaxin, and IFNa2 were identified in saliva from infected patients allowing for differentiation between bacterial and viral infections. These data suggest that saliva can serve as a suitable, easily obtained source for rapid biomarker identification, which, when combined with standard of care, can lead to early diagnosis and improved prognosis for treatment of infected military personnel. Continued study of novel methodologies for rapid identification of biomarkers associated with microbial infections, may lead to improved treatment protocols, improved prognosis, and an overall decrease in the use of unnecessary antibiotics.

15. SUBJECT TERMS
innate immune biomarkers, saliva, cytokines, chemokines, bacterial infections, viral infections, rapid identification

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